International Journal of Agricultural
Science and Research (IJASR)
ISSN (P): 2250-0057; ISSN (E): 2321-0087
Vol. 7, Issue 4, Aug 2017, 195-202

CHARACTERIZATION OF *RHIZOBIUM* ISOLATED FROM ROOT NODULE OF PEA (*PISUM* SATIVUM L.)

ABDOUSLAM M. RASSEM[1] & ARUN A. DAVID[2]

[1]*Research Scholar Department of Soil Science, Sam Higginbottom University of Agriculture, Technology & Sciences Allahabad, U.P., India*

[2]*Associate Professor Department of Soil Science, Sam Higginbottom University of Agriculture, Technology & Sciences Allahabad, U.P., India*

ABSTRACT

Studies were conducted on root nodules, collected from young and healthy seedling of pea, at research farm of soil science, Naini Agricultural Institute, Allahabad (SHUATS), U.P, India. The isolates were further subjected to morphological, biochemical and physiological (stress tolerance) characterization; three Rhizobium strains were isolated from the root nodule of field pea and characterized by standard biochemical tests. Isolated Rhizobia was fast grower, all strains were gram-negative rod and creamy color colony. Rhizobium strains showed difference in chemical test and urea hydrolysis, and the strains utilized glucose and mannitol as fermentation sugar, growth of Rhizobium strains tolerated to stress as temperature were at 20°C and 28°C, also observed well growth of sinorhizobium meliloti and rhizobium pusense strains at 2 % NaCl, while leguminosarum strain was grown at 3 % NaCl, and all strains were grown at pH 6 and pH 7. Optimum conditions help to enhance plant and super strain for fixed nitrogen in nodules of field pea crop.

KEYWORDS: Rhizobium Characterization, Biochemical Tests, Field Pea & Stress Tolerance

Received: Jun 06, 2017; **Accepted**: Jun 20, 2017; **Published**: Jul 12, 2017; **Paper Id:** IJASRAUG201724

Original Article

INTRODUCTION

The soil includes many types of microorganisms such as bacteria, actinomycetes, fungi, and algae, which are important because, they affect the physical, chemical, and biological properties of soil and growth plant amongst the soil bacteria, a unique group called, rhizobia having a beneficial effect on the growth of plants. It can live either in the soil or within the root nodules of host legumes. The bacteria colonize within root nodules, where it converts atmospheric nitrogen to ammonia and provides organic nitrogenous compounds to the plants. In legumes and a few other plants, the bacteria live in small outgrowths on the roots called nodules, within these nodules, the bacteria do nitrogen fixation, and the plant absorbs the ammonia (Oblisami, 1995)

Legumes have been used in agriculture since ancient times, and legume seeds or pulses were among the first source of human food and their domestication. Legume plant possesses a unique ability to establish a symbiosis with nitrogen-fixing bacteria of the family *rhizobiaceae*. The bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Rinorhizobium* and *Mesorhizobium* (Willams, 2006), which are collectively referred to as *rhizobia*, are able to form nodules on their host plants, and the fixation of N_2 by legumes plays a key role in agricultural sustainability. Moreover, the further assessment of *rhizobium* genetic diversity is contributing both to the world wide knowledge of biodiversity of soil micro-organisms and to the usefulness of *rhizobium* collections, and it is developing long-term strategies to increase contributions of legume-fixed to

agricultural productivity. Low soil pH does not allow the *rhizobium* cells to survive inadequate numbers in free-living state, consequently it becomes inevitable to inoculate the crop in adequate *rhizobium* (Deka *et al*., 2006). Increasing salt concentration may have detrimental effects on *rhizobium* population (Singleton *et al*., 1992) among the factors that affect growth and survival of these genera. There have been reports of the detrimental effects of salt stress on plant growth, multiplication of *rhizobia*, nodulation and nitrogen fixation (Embalomatis, *et al*., 1994). The harmful effect of salinity on *rhizobia* and *bradyrhizobia* may be due to direct specific ion effects or to the indirect effect of salinity, by raising the pH value and decreasing osmotic potential. Salt tolerant *rhizobia* may have the potential to improve yield of legumes under salinity stress (El-Mokadem, 1991). *rhizobium* inoculation increases nodule biomass, thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation (Adewusi *et al*., 2008). Various researches demonstrated the ability of *rhizobium* to colonize roots of nonlegumes (Matiru *et al*., 2004) and act as phytohormone producer, phosphate solubilizer and to some extent, as nitrogen fixer (Afzal *et al*., 2008). Bio-fertilizer promotes plant growth and productivity, which has internationally been accepted as an alternative source of chemical fertilizer. *Rhizobacteria* effectively colonize plant root and increases plant growth by production of various plant growth hormones, P-solubilizing activity, N_2 fixation and biological control activity (Deshwal *et al.,* 2011). Modulated wild legumes have potential for nitrogen fixation, reforestation and to control soil erosion (Elsoni *et al*., 2011).

MATERIALS AND METHODS

Study Area

This study was conducted in the district during 2015-16, the sample was collected from the research farm of soil science, Naini Agricultural Institute (NAI), Allahabad (SHUATS) at a latitude of 20^0 15^0 North and longitude of 60^0 3^0East, and at an altitude of 98 meters, above mean sea level (MSL).

Procedure

Three samples of root nodules from field pea (*Pisum sativum* L.) were collected randomly from different localities. From each plant sampled, three to four nodules were at random excised and healthy root nodules were washed with tape water thrice, before streaking on agar plate as described by (Vincent, 1970; Ben-Gweirif *et al.,* 2005). The nodules were sterilized externally, using 95 % alcohol for 1-4 minute, followed by washing with calcium hypochlorite solution (10g/150 ml distilled water) and crushing in a drop of sterile water. A loopful ground material was transferred to 5 ml of sterile water, of which, 0.1 ml sample was spread onto the surface of yeast extract manitol agar (YEMA). Plates were then incubated at 28^{o}C for 48 hours; well isolated typical single colonies were re-streaked on freshly prepared YEMA plates, in order to obtain pure cultures to study morphological, cultural and biochemical characteristics.

Morphological Characteristics

Morphological characteristics of colour, shape, morphology and colony were observed under low power microscope, similarly using gram staining technique, as described by (Arora, 2003).

Biochemical Tests

All the collected samples were processed through different biochemical tests for identification, and studied characters of strains isolated and biochemical tests were carried out viz, catalase test (Facklam and Elliott, 1995), indole production test (MacFaddin, 2000) methyl red and vogas proskauer test (Vogesand Proskauer, 1898), citrate utilization test as described by (Claus, 1989) and starch hydrolysis test as mentioned by (Arora, 2003), urease test (Collins and Patricia,

1995), casein hydrolysis test (Salisbury *et al.*, 1972) and mannitol fermentation as mentioned by (Cappuccino, 2008)

Salt, pH and Temperature Tolerance

Physiological test range of salt, pH and temperature tolerance for growth optimum, the ability of the isolated *rhizobium* strain to grow in different concentration of salt was tested by streaking them on YEM medium containing 1.0 %, 2.0 %, 3.0 %, 4.0 %, 4.5 % and 5.0 % (w/v) NaCl. After five days of incubation, the absorbance of the resultant growth of the bacteria in the broth tubes was measured using a spectrophotometer at a wavelength 540 nm, as described by (Vincent, 1970; Rafiq, 1997). The differences in pH tolerance were adjusted with the pH to 4.0, 5.0, 6.0, 7.0 and 8.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates were used as controls. The difference in the range of growth temperature was investigated by incubation of bacterial cultures at 5°C, 10°C, 15°C, 20°C, 28°C, 38°C, 40°C, 45°C and 50°C. Control plates were incubated at 28°C, after incubation, the absorbance of the result growth of the bacteria in the broth tubes was measured using a spectrophotometer, at a wavelength 540 nm as described by (Vincent, 1970; Gao *et al*., 1994; Mensah *et al*., 2006).

RESULTS AND CONCLUSION

In the study, three strains were isolated (Table 1) from root nodules of (*Pisum sativum* L.) collected from different locations in Allahabad

Table 1: Isolates of Bacteria from Root Nodules of Pea

SR. No.	Name of the Isolate	Location of Isolates
1	RS_1- [*Leguminosarum*]	Soil Science Research Farm
2	RS_2-[*Sinorhizobiu meliloti*]	Soil Science Research Farm
3	RS_3- [*Rhizobium pusense*]	Soil Science Research Farm

All strains tested were found to have rod colonies with regular borders, flat in elevation, creamy in colour after 3 to 5 days of growth on YEMA at 28°C, on the basis of morphological isolates (Table 2). The colony were observed under low power microscope, by using a gram staining technique as described by (Arora, 2003), and pink colour gram negative rods were observed.

Three samples of root nodules from pea (*Pisum sativa* L.) were found, one strain positive for the presence *of legminosurm.* After screening through a series of various biochemical tests, one strain was characterized biochemically as *legminosurm* gram negative rods with circular, raised and smooth edges colony observed. These findings are in line with (Hussain *et al.*, 2002; Oblisami, 1995), who also isolated the *rhizobium legminosurm* from pea with same Characteristics.

All the samples were also streaked on yeast extract mannitol (YEM) in selective media, for further confirmation. Similarly, the positive samples from all target areas showed hazy appearance in the motility media, and also were positive for catalase, glucose fermentation, mannitol fermentation tests, indole production test and starch hydrolysis test, and were found negative for methyl red (MR), voges-proskauer (VP), urea hydrolysis tests, casein hydrolysis test and citrate utilization test. These findings are in close agreement with (Elsheikh and Wood, 1989; Javed and Asghari, 2008), who also characterized the *rhizobium* from soil, and sunflower root nodules with the same positive biochemical tests. Similarly, (Oblisami, 1995) also studied the nodulation pattern in forage legume bacteria, by screening through the same tests results, and (Singh *et al.*, 2008) characterized *rhizobium* strain from the roots of *Trigonella foenumgraecum.*

Table 2: Morphological and Biochemical Characterization of Rhizobium Isolated From Pea

S.No.	Particulars	Strain of *Rhizobium*		
		RS_1	**RS_2**	**RS_3**
1	Gram stain –reaction	-	-	-
2	Colony morphology	**rod**	**rod**	**Rod**
3	Colony colour	**Creamy**	**Creamy**	**Creamy**
4	Methyl-red (MR)	-	-	+
5	Voges-Proskauer tests (VP)	-	-	-
6	Citrate utilization test	-	+	-
7	Casein hydrolysis test	-	-	-
8	Catalase Test	+	+	+
9	Indole production test	+	-	-
10	Urease test	-	+	+
11	Starch hydrolysis test	+	+	+
12	Glucose fermentation	+	+	+
13	Mannitol fermentation	+	+	+

The motility media also were positive for RS_2, and RS_3were positive for catalase, glucose fermentation, mannitol fermentation tests, urea hydrolysis tests and starch hydrolysis test and only positive RS_3 for methyl red. RS_2 and RS_3 were found negative for methyl red (MR), voges-proskauer (VP), urea hydrolysis tests, casein hydrolysis test, casein hydrolysis test and indole production test as shown in table (2).

The physiological traits of three strains were summarized in Table 3 and 4, also in Figure 1, 2 and 3. The table 3 shows effected temperature tolerance on the strain, the maximum temperature where, more of the isolates' growth was 28°C. The percentage of isolates grew well at 20 and 28°C, and differentiation according to temperature tolerance started at 28°C and all the isolates were surviving at above temperature, and very low of the strains showed growth at temperature 5°C and50°C.

Table 3: Effected of Temperature on Rhizobium Isolated From Pea

Strains	Absorbance of Strain *Rhizobium*								
	5°C	**10°C**	**15°C**	**20°C**	**28°C**	**38°C**	**40°C**	**45°C**	**50°C**
RS_1	0.07	0.13	0.28	0.37	0.48	0.36	0.28	0.20	0.16
RS_2	0.08	0.20	0.37	0.42	0.51	0.25	0.20	0.17	0.12
RS_3	0.09	0.23	0.40	0.57	0.63	0.57	0.52	0.47	0.39

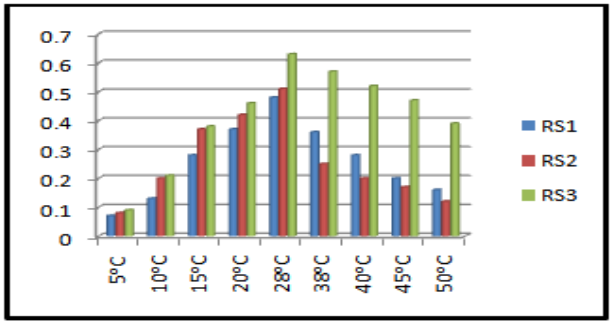

Figure 1: Effected of Temperature on Rhizobium Isolated From Pea

All *rhizobia* isolated were able to grow in all ranges of the various sodium chloride concentrations tested. The ability to tolerate high salt is far greater for the native strains, which grew less in above 2 % sodium chloride concentrations of the medium (Table 4). But, the native *rhizobium* strains RS_1 isolated were able to grow variably

throughout the different sodium chloride range tested from 3 % to 4 %, indicating the fact that, native *rhizobium* strains were more adapted to soils and highly concentrated with various forms of cations and anions, All strains were grown at controlled cultural conditions (0.01 % NaCl, pH 7). All isolates strains RS_2 and RS_3 showed growth at 1.0 % and decreased growth with increasing NaCl concentration, however, strain 1 showed good growth at 3.0 % NaCl concentration.

Table 4: Tolerance of Rhizobium Strains to pH and NaCl Concentrations

Strains	pH					NaCl (w/v)					
	4	5	6	7	8	1.0	2.0	3.0	4.0	4.5	5.0
RS_1	0.89	0.90	0.96	0.98	1	0.31	0.28	0.53	0.50	0.21	0.17
RS_2	0.30	0.33	0.40	0.44	0.44	0.55	0.48	0.35	0.32	0.25	0.20
RS_3	0.75	0.80	0.84	0.90	0.93	0.42	0.37	0.34	0.32	0.27	0.22

A growth strain in a different range of pH was noted, when pH at 4 of all strains RS_1, RS_2 and RS_3 decreased growth. Optimum pH range for rhizobia was between pH 6 and pH 7, which was increasing growth as shown in table 4. No *rhizobium* strains were able to grow at pH of the medium adjusted to 4, all the rest, including *rhizobia* were able to grow on the medium adjusted to pH 7 (Table 4). In the soil environment, the condition is highly different. All the native *rhizobium* strains were able to survive well in the various soils adjusted to pH 4 up to 7.

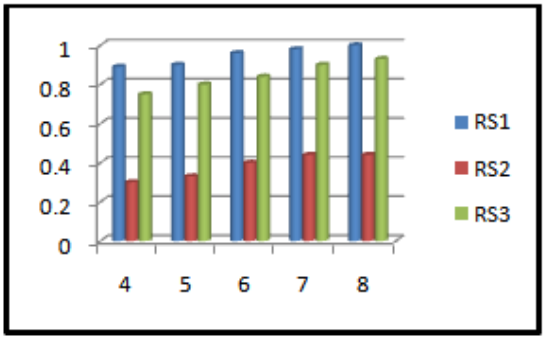

Figure 2: Tolerance of Rhizobium Strains to Ph

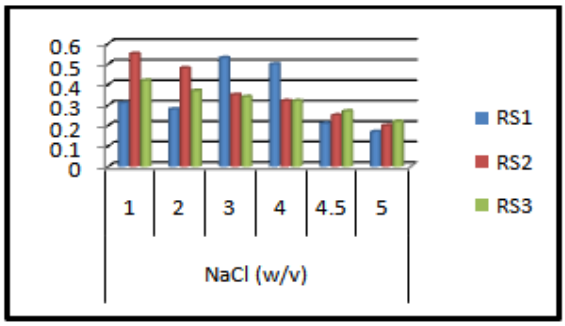

Figure 3: Tolerance of Rhizobium Strains to Nacl Concentrations

Table 5: Identified of Rhizobium Species Isolated By 16S Rrna Amplification

Sr. No.	Isolates	Type of *Rhizobium*	Name of *Rhizobium*	Gene Bank Accession No Sequence ID	Query ID
1	RS_1	*Leguminosarum*	ABDR1	LC176423.1	151659
2	RS_2	*Sinorhizobium meliloti*	ABDR2	LC176424.1	137359
3	RS_3	*Rhizobium pusense*	ABDR3	LC176425.1	180517

Table 5 shows results of PCR test of strains RS_1, RS_2 and RS_3 and 16S rRNA gene sequences by using NCBI BLAST for a known type of *rhizobium* that was isolated from a nodule of root Pea. One strain was *leguminosarm, Sinorhizobium meliloti* and *Rhizobium* pusense by user ID sequences as show in table i.e. the form of gene sequences in Gene bank

ACKNOWLEDGEMENT

I am highly indebted to my advisor for his guidance and constant supervision as well as for providing necessary information regarding the study. I express heartfelt thanks to the Hon'ble vice chancellor, HOD and advisor, department of soil science, (SHUATS) Allahabad, U. P., and Cytogene Research and Development Indira Nagar, Lucknow for providing all necessary facilities.

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